Services Provided in Support of the Planetary Quarantine Requirements of the National Aeronautics and Space Administration under Contract R-137

Report No. 26
April - June 1969

Applied Microbiology and
Planetary Quarantine Section
Phoenix Laboratories
Ecological Investigations Program
National Communicable Disease Center
Public Health Service
U.S. Department of Health, Education, and Welfare
Phoenix, Arizona

Contributors:

Biophysics Unit	Experimental Microbiology Unit	Spacecraft Bioassay Unit
N. Petersen	W. Bond	J. Puleo
J. Marshall	L. Carson	G. Oxborrow
		N. Fields

Report submitted by:

Martin S. Favero, Ph.D., Chief
Applied Microbiology and
Planetary Quarantine Section

Report reviewed and forwarded by:

James E. Maynard, M.D., Chief
Ecological Investigations Program
Phoenix Laboratories

July 1969

1. Studies for determining a frequency of D_{125C} values for spores isolated from spacecraft in residence at Cape Kennedy were continued. As reported last quarter, 145 isolates from the Jet Propulsion Laboratory's (JPL) spore assays conducted on Mariner 69 were received from Cape Kennedy. The isolates were grown on TAM Sporulation Agar (Difco) supplemented with 20 ppm magnesium sulfate and 80 ppm calcium chloride (Report No. 25). A total of 115 spore crops were prepared and titered. The remainder of the 145 isolates either were mixed cultures, other than gram positive rods, or did not sporulate on TAM. Of the 115 crops prepared, twelve were discarded because too few spores were formed for adequate testing.

   A multiple replicate unit testing system was conducted in the following manner. For each spore crop, each of 33 sterile 1/2 x 1/2" stainless steel strips was inoculated with 5×10^5 to 5×10^6 spores (suspended in ethanol) using a dropper pipette (Report No. 25). The test strips were held under vacuum over silica gel for 16 hours prior to assay. Three unheated strips inoculated with each suspension were processed in the standard manner (Report No. 19) and plated in triplicate with Trypticase Soy Agar (TSA) supplemented with 0.1% soluble starch and 0.2% yeast extract (Report No. 25) in order to obtain N_o (control) values. The remaining 30 strips were suspended in 6 quintuplicate sets in the modified 125 C forced air oven (Report No. 25). Sets were removed at 40 minute intervals (e.g., 0, 40, 80, 120, 160 and 200) and allowed to cool to room temperature (2 min.) in a horizontal laminar flow cabinet. Strips were then removed from their hangers and placed in tubes of TSA-B (broth made according to the formula of TSA minus agar and supplemented with 0.1% soluble starch and 0.2% yeast extract). Visible growth in the tubes was recorded after a maximum incubation of 2 weeks at 32 C. D_{125C} values were calculated from fractional-replicate-unit-negative (FN) data using a most probable number (MPN) technique described by Pflug and Schmidt (Pflug, I. J., and C. F. Schmidt. Thermal destruction of microorganisms. In: Disinfection, Sterilization, and Preservation. C. A. Lawrence and S. S. Block, Lea and Febiger, Philadelphia, 1968, p. 63-105).

   Preliminary tests were conducted to compare N_o values based on colony counts from pour plates (supplemented TSA) and those based on an MPN system (supplemented TSA-B). Decimal dilutions were prepared from inoculated strips and plated with the solid medium. In addition 1-ml portions of each dilution were pipetted into five tubes of TSA-B. Plates and tubes were incubated at 32 C. The results of five trials (Table 1) showed that there were no marked differences in spore counts between the two methods.

   Another series of parallel tests was performed in order to compare D_{125C} values obtained by both assay methods. The spores employed were Bacillus subtilis var. niger in 95% ethanol (BgHA, "old" standard crop). Pour plates were prepared in the usual fashion (Report No. 19) using supplemented TSA. D_{125C} values were determined from the slopes of best-fit lines through survivor points. The FN-MPN system was used as described above. Results of three trials are shown in Table 2. The higher D_{125C} values obtained by the FN-MPN system could have been due to factors inherent in the calculations involved and/or actual increased recovery of heat-injured spores by the liquid system. Attempts to resolve the inconsistencies in these data will be made during the next quarter.

Figure 1 shows the frequency distribution of D_{125C} values obtained for the 103 spore isolates from the Mariner 69 spacecraft. The values ranged from less than 5 minutes to a maximum of 58 minutes. The spore level on the Mariner 69 was approximately 1×10^4 (R. H. Green, Jet Propulsion Laboratory, personal communication). Assumming that the spore population on the spacecraft had this frequency distribution of D_{125C} values, the survival curve of this population at 125 C would be as shown in Figure 2. Using the mean of these D_{125C} values as the basis of a sterilization cycle would not be realistic in the sense that the slope of the spore population's survival curve is determined almost exclusively by those organisms which have a high degree of resistance to dry heat, even though they represent a small portion of the population.

It is emphasized that these data should be considered "relative" and not "absolute". It has been shown previously that the subculturing of naturally occurring spores results in a significant loss of dry heat resistance. Although the exact reasons for this phenomenon are not fully understood at the present time, the composition of the sporulation medium obviously affects the degree of dry heat resistance obtained with a particular spore crop. Naturally occurring spores lose a significant degree of dry heat resistance when sporulated on the TAM sporulation agar. However, it appears that the liquid medium of Lazzarini and Santangelo (SSM-10) (Report No. 25), which was used by the Jet Propulsion Laboratory (R. H. Green, personal communication) with the same Mariner 69 isolates, is one in which this loss of resistance does not occur to the same degree. Consequently, it may be speculated that the frequency distribution of D_{125C} values of these spore isolates will more closely reflect the actual dry heat resistance of the spores prior to subculture. Studies will be conducted during the next quarter to compare dry heat resistances of naturally occurring spores and the same populations subcultured using the SSM-10 medium.

2. At the request of the Planetary Quarantine Officer studies were initiated in March to determine whether viruses could be recovered from space hardware. Specified surfaces of Apollo spacecraft were sampled using moistened swabs. Swabs from 32 areas, divided among four tubes, constituted one sample. The swabs were placed in Basal Medium Eagles containing 1% calf serum, double strength antibiotics and 0.5% gelatin. These were frozen immediately on dry ice and kept frozen during shipment to the Phoenix Laboratories where they were processed by the Microbiology Unit of the Disease Investigations Section. The swabs were thawed, insonated for 2 minutes and either inoculated onto tissue cultures or stored at -70 C until examined.

   For enteric viruses, primary monkey kidney, primary human amnion, continuous human embryonic kidney and HeLa cell culture systems were used. Five passages were made in each cell culture system and specimens were considered negative for viruses if no cytopathology was evident by the fifth passage.

   For upper respiratory viruses, continuous HEp-2 and WI:38 cell culture systems were used and four passages were made before samples were considered negative.

Samples from the following spacecraft were examined:

Apollo 10 - Command Module interior (swabs taken 3/7/69)

Apollo 11 - Lunar Module 5, ascent interior (3/28/69)

Apollo 11 - Command Module interior (4/11/69)

Apollo 11 - Command Module 107 (4/28/69)

Apollo 12 - Lunar Module 6, ascent interior (5/9/69)

Apollo 12 - Lunar Module 6, ascent interior (6/9/69)

To date no enteroviruses or upper respiratory viruses have been detected.

3. Data from the identification of 4,276 aerobic mesophilic microorganisms isolated from the Apollo 8 and 9 spacecraft are presented in Tables 3 and 4. Most of the contaminants isolated from the Command Modules (CSM-103 and CSM-104) and Lunar Modules (LM-3) were those microorganisms considered to be indigenous to humans (i.e., Staphylococcus spp. and Micrococcus spp.; ca. 89-99%). On the other hand, microorganisms associated with soil and dust (Bacillus spp., molds and actinomycetes) were found to constitute a relatively high percentage of the contaminants recovered from the Instrument Units (I.U.; ca. 32%) and the Saturn S-4B stage (S-4B; ca. 27%). This marked difference in general types of microorganisms detected in the various spacecraft components could have been due to differences in environmental control practiced during assembly and testing operations.

The Apollo 10 spacecraft was studied during its residency at Launch Complex 39B. The levels of microbial contamination present on the CSM-106, I.U., S-4B, and the Spacecraft Lunar Module Adapter (SLA) are presented in Table 5. Contamination levels for the CSM and SLA remained basically the same throughout the study period. The interior surfaces of the SLA were found to contain relatively low levels (ca. 200/ft^2) of microbial contamination. Although the levels of aerobic mesophilic microorganisms were similar among the CSM, I.U. and S-4B (ca. $10^4/ft^2$), the concentration of bacterial spores on the CSM was approximately 1 log lower than the I.U. and S-4B.

Table 6 shows the quantitative data collected on the interior and exterior surfaces of the ascent and descent stages of the Lunar Module 4 (LM-4). The levels of microbial contamination in the interior of the LM-4 were found to be one log higher than those in the LM-3 (Report No. 25) and 1-3 logs higher than the levels detected on the exterior surfaces of the ascent and descent stages (LM-4). The continuous purging of the exterior surfaces with large volumes of filtered air probably accounted for the reduction in exterior contamination levels. This phenomenon also was observed with the LM-3.

The percentages of aerobic spores and molds on the interior surfaces of the Apollo 10 Command Module (CSM-106), the I.U., S-4B and the SLA are shown in Table 7. The I.U. and S-4B contained significantly higher levels of aerobic

spores and molds than the CSM, I.U. and S-4B portions of both Apollo 8 and 9 (Reports Nos. 24 and 25). The SLA also contained a relatively high percentage of aerobic spores. The percentage of spores and molds on the interior surfaces of the LM-4 (Table 8) was lower than those observed on the interior surfaces of the CSM-106. The exterior surfaces of the ascent and descent stages of LM-4 showed a higher percentage of spores than was observed on the exterior surfaces of LM-3 (Report No. 25).

Studies were continued on the Apollo 11 spacecraft. The levels of microbial contamination of the interior surfaces of LM-5 (Table 9) appeared to be higher than those on LM-3 (Report No. 25) and similar to those obtained for LM-4. Levels on the exterior surfaces of the ascent and descent stages were similar to those for LM-4.

Studies were initiated for the Apollo 12 spacecraft. Results of the preliminary sampling of the Lunar Module 6 (LM-6) are presented in Table 10. The initial data appear to be similar to those obtained with LM-5.

4. A total of 2,000 bacterial cultures was isolated from the Apollo 10 spacecraft. These isolates are being identified and will be reported during the next quarter. All data generated from this effort will be stored on IBM cards and ultimately on the computer at Cape Kennedy. Pertinent protocols, data forms, shipping procedures were finished. It is estimated that the data on isolates from Apollo 11 will be available in complete form to NASA headquarters by August 10, 1969.

5. In accordance with a request from the Planetary Quarantine Officer, the Biosatellite Spacecraft (Primate Mission Flight D) was sampled for microbial contamination one day prior to launch (June 27). Results will be reported in the next quarter.

TABLE 1. COMPARISON OF THE POUR PLATE AND MPN TECHNIQUES FOR ENUMERATING SPORES.

Spore Isolate[1]	Number of Spores per Strip	
	Pour Plate[2]	MPN[3]
1	1.0×10^6	9.9×10^5
2	2.5×10^6	1.4×10^6
3	2.2×10^6	1.7×10^6
4	1.7×10^6	2.3×10^6
5	1.6×10^6	2.3×10^6

[1] Spores were suspended in 95% ethanol.

[2] Pour Plate: The medium used was Trypticase Soy Agar (TSA) supplemented with 0.1% soluble starch and 0.2% yeast extract. All pour plates were overlayed to inhibit spreaders. Counts were made after 24, 48, and 72 hours incubation at 32 C.

[3] Most-Probable-Number: The medium employed was Trypticase Soy Broth (TSA-B) made according to the formula of TSA minus agar and supplemented with 0.1% soluble starch and 0.2% yeast extract. Tubes were incubated for 2 weeks at 32 C.

TABLE 2. COMPARATIVE D_{125C} VALUES FOR BACILLUS SUBTILIS VAR. NIGER SPORES USING TWO ASSAY METHODS.

Test	N_o (Unheated Control) Spores per Strip	D_{125C} (Min) Standard Method[1]	D_{125C} (Min) FN-MPN[2]
1	1.0×10^6	15	24
2	1.0×10^6	15	24
3	7.2×10^6	15	25.5

[1] D_{125C} values were determined from the best fit line of survivor points which were obtained by pour plates using supplemented TSA.

[2] D_{125C} values were determined from fractional-replicate-unit-negative data (see text) using TSA-B.

TABLE 3. TYPES OF AEROBIC MESOPHILIC MICROORGANISMS ISOLATED FROM THE APOLLO 9 SPACECRAFT.

Microorganisms	Command Module 104 (Interior)		LM-3 Ascent (Interior)		LM-3 Ascent (Exterior)		LM-3 Descent (Exterior)		Instrument Unit (Interior)		S-4B (Interior)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Staphylococcus epidermidis	121	16.7	337	24.4	156	15.0	188	18.4	1	1.8	0	0.0
Staphylococcus aureus	0	0.0	0	0.0	4	0.4	1	0.1	2	3.5	0	0.0
Micrococcus spp.	365	50.6	834	60.3	648	62.4	617	60.3	9	15.8	6	11.5
Sarcina spp.	0	0.0	6	0.4	2	0.2	5	0.5	0	0.0	0	0.0
Gaffkya spp.	14	1.9	37	2.7	31	3.0	21	2.1	0	0.0	0	0.0
Streptococcus spp.	1	0.1	4	0.3	3	0.3	8	0.8	0	0.0	0	0.0
Corynebacterium-Brevibacterium group	212	29.3	126	9.1	98	9.4	82	8.0	15	26.3	26	50.0
Gram negative rods	1	0.1	9	0.6	7	0.7	28	2.7	10	17.5	6	11.5
Bacillus spp.	8	1.1	10	0.7	35	3.4	40	3.9	9	15.8	12	23.2
Yeasts	1	0.1	1	0.1	14	1.4	4	0.4	2	3.5	0	0.0
Molds	1	0.1	19	1.4	35	3.4	26	2.5	7	12.3	2	3.8
Actinomycetes	0	0.0	0	0.0	4	0.4	3	0.3	2	3.5	0	0.0
TOTAL	724	100.0	1,383	100.0	1,037	100.0	1,023	100.0	57	100.0	52	100.0

TABLE 4. TYPES OF AEROBIC MESOPHILIC MICROORGANISMS ISOLATED FROM THE APOLLO 8 SPACECRAFT.

Microorganisms	Command Module 103 (Interior) No.	%	Instrument Unit (Interior) No.	%	S-4B (Interior) No.	%
Staphylococcus epidermidis	204	13.1	16	3.2	4	0.8
Staphylococcus aureus	3	0.2	1	0.2	0	0.0
Micrococcus spp.	1,087	69.6	299	58.9	317	61.9
Sarcina spp.	10	0.6	5	1.0	0	0.0
Gaffkya spp.	47	3.0	9	1.8	15	2.9
Streptococcus spp.	38	2.4	14	2.8	18	3.5
Corynebacterium-Brevibacterium group	139	9.0	109	21.5	114	22.3
Gram negative rods	5	0.3	6	1.2	3	0.6
Bacillus spp.	17	1.1	20	3.9	15	2.9
Yeasts	0	0.0	2	0.4	0	0.0
Molds	10	0.6	20	3.9	23	4.5
Actinomycetes	1	0.1	6	1.2	3	0.6
TOTAL	1,561	100.0	507	100.0	512	100.0

TABLE 5. LEVELS OF MICROBIAL CONTAMINATION DETECTED ON THE APOLLO 10 COMMAND MODULE (CSM-106), INSTRUMENT UNIT, SATURN S-4B, AND THE SPACECRAFT LUNAR MODULE ADAPTER (SLA).

Source	Date Sampled	Area Sampled[2] (sq. in.)	Microorganisms per square foot			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Command Module[1] (CSM-106)	5-01-69	60	26,755	15,739	202	12
	5-09-69	60	20,866	12,254	187	25
	5-16-69	60	16,157	10,757	120	25
Instrument Unit[1]	5-01-69	60	8,309	1,613	2,563	230
	5-09-69	60	6,595	2,203	1,253	133
	5-15-69	60	29,779	4,219	1,973	446
S-4B[1]	5-01-69	60	6,926	936	2,520	403
	5-09-69	60	38,851	7,099	3,024	230
	5-15-69	60	16,891	2,002	3,643	331
SLA[1]	5-01-69	60	158	0	12	0
	5-11-69	60	317	0	12	0
	5-14-69	60	173	72	12	48

[1] Samples were taken from the interior surfaces of the spacecraft located at Launch Complex 39B.

[2] Swab-rinse technique

TABLE 6. LEVELS OF MICROBIAL CONTAMINATION DETECTED ON LUNAR MODULE 4 (APOLLO 10).

Source	Date Sampled[1]	Area Sampled[2] (sq. in.)	Microorganisms per square foot			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Lunar Module 4 (Interior)	5-01-69	60	211,378	195,307	403	25
	5-11-69	60	188,597	41,299	360	25
	5-14-69	60	141,970	78,120	360	61
Lunar Module 4 Ascent Stage (Exterior)	5-01-69	60	9,302	1,670	158	36
	5-11-69	60	878	187	130	0
	5-14-69	60	4,709	1,555	173	25
Lunar Module 4 Descent Stage (Exterior)	5-01-69	60	34,459	27,187	403	108
	5-11-69	60	5,472	4,392	418	55
	5-14-69	60	7,762	2,203	720	0

[1] Samples were taken from Lunar Module 4 while it was located at Launch Complex 39B.

[2] Swab-rinse technique.

TABLE 7. COMPARATIVE LEVELS OF AEROBIC BACTERIAL SPORES AND MOLDS DETECTED ON THE INTERIOR SURFACES OF THE APOLLO 10 COMMAND MODULE (CSM-106), INSTRUMENT UNIT, SATURN S-4B, AND SPACECRAFT LUNAR MODULE ADAPTER (SLA).

Source	Date Sampled[1]	Area Sampled[2] (sq. in.)	Percent[3]	
			Aerobic Bacterial Spores	Molds
Command Module (CSM-106)	5-01-69	60	0.75	0.05
	5-09-69	60	0.90	0.0
	5-16-69	60	0.74	0.0
Instrument Unit	5-01-69	60	30.85	3.12
	5-09-69	60	19.00	9.39
	5-15-69	60	6.63	2.85
S-4B	5-01-69	60	36.38	3.33
	5-09-69	60	7.78	1.04
	5-15-69	60	21.57	3.67
SLA	5-01-69	60	7.59	0.0
	5-11-69	60	3.79	4.54
	5-14-69	60	6.94	0.0

[1] Samples were taken from the interior surfaces of the spacecraft while they were located at Launch Complex 39B.

[2] Swab-rinse technique.

[3] Percentage of total aerobic mesophilic microorganisms.

TABLE 8. COMPARATIVE LEVELS OF AEROBIC BACTERIAL SPORES AND MOLDS DETECTED ON THE SURFACES OF THE LUNAR MODULE 4 ASCENT AND DESCENT STAGES.

Source	Date Sampled[1]	Area Sampled[2] (sq. in.)	Percent[3]	
			Aerobic Bacterial Spores	Molds
Lunar Module 4 (Interior)	5-01-69	60	0.19	0.0
	5-11-69	60	0.19	0.02
	5-14-69	60	0.25	0.0
Lunar Module 4 Ascent Stage (Exterior)	5-01-69	60	1.70	0.31
	5-11-69	60	14.81	0.0
	5-14-69	60	3.67	0.61
Lunar Module 4 Descent Stage (Exterior)	5-01-69	60	1.17	0.17
	5-11-69	60	7.64	2.37
	5-14-69	60	9.28	2.41

[1] Samples were taken while the Lunar Module was located at Launch Complex 39B.

[2] Swab-rinse technique.

[3] Percentage of total aerobic mesophilic microorganisms.

TABLE 9. LEVELS OF MICROBIAL CONTAMINATION DETECTED ON LUNAR MODULE 5 (APOLLO 11).

Source	Date Sampled	Area Sampled[1] (sq. in.)	Microorganisms per square foot			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Lunar Module 5 Ascent Stage (Interior)	2-27-69[2]	60	47,866	12,643	48	25
	4-04-69[2]	45	235,987	74,419	108	55
	5-12-69[3]	60	125,510	63,274	317	36
	5-26-69[4]	60	75,758	22,363	230	12
	6-18-69[4]	60	103,234	54,346	302	72
Lunar Module 5 Ascent Stage (Interior)	2-27-69[2]	60	4,637	1,152	48	12
	4-18-69[3]	60	1,282	302	144	216
	5-06-69[3]	60	4,464	3,355	61	25
	5-26-69[4]	60	4,939	878	36	0
	6-18-69[4]	60	1,253	331	120	25
Lunar Module 5 Descent Stage (Exterior)	4-18-69[2]	60	52,504	23,947	374	84
	5-06-69[3]	60	9,533	5,083	144	0
	5-26-69[4]	60	1,116	1,699	61	36
	6-18-69[4]	60	3,427	1,282	133	25

[1] Swab-rinse technique.

[2] Samples were taken while the modules were located in the Manned Spacecraft Operations Building.

[3] Samples were taken while the modules were located in the Vehicle Assembly Building.

[4] Samples were taken while the modules were located at Launch Complex 39A.

TABLE 10. LEVELS OF MICROBIAL CONTAMINATION DETECTED ON LUNAR MODULE 6 (APOLLO 12).

Source	Date Sampled[1]	Area Sampled[2] (sq. in.)	Microorganisms per square foot			
			Aerobes	Anaerobes	Aerobic spores	Anaerobic spores
Lunar Module 6 Ascent Stage (Interior)	4-22-69	60	6,595	3,600	173	0
	6-19-69	60	96,898	48,082	691	61
Lunar Module 6 Ascent Stage (Exterior)	4-22-69	60	2,966	274	72	12
	5-22-69	60	9,734	8,611	84	97
	6-19-69	60	10,944	5,126	120	36
Lunar Module 6 Descent Stage (Exterior)	4-22-69	60	4,954	1,498	158	25
	5-22-69	60	22,363	9,115	274	245
	6-19-69	60	50,760	29,088	403	72

[1] Samples were taken while the modules were located in the Manned Spacecraft Operations Building.

[2] Swab-rinse technique.

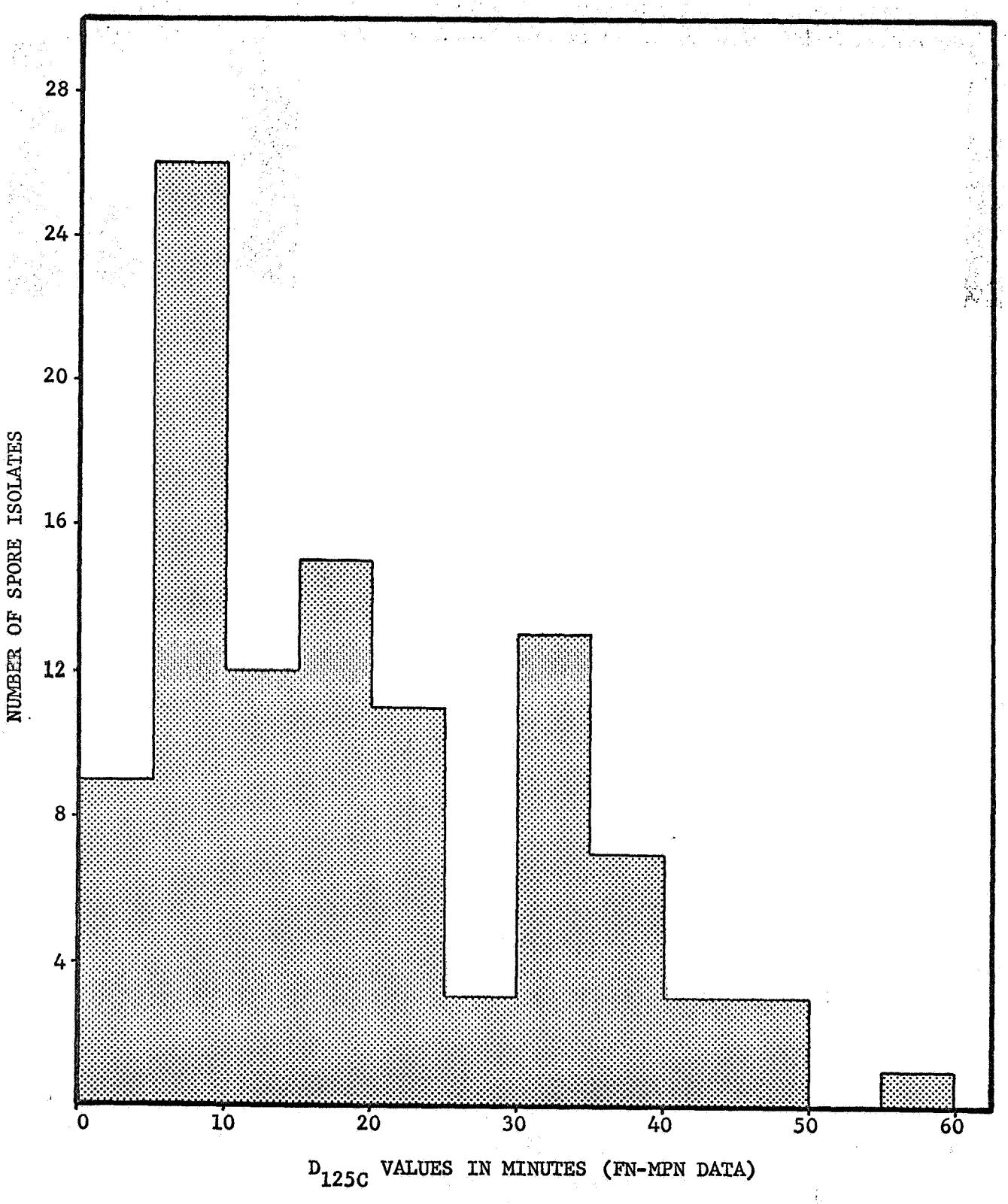

FIGURE 1. FREQUENCY DISTRIBUTION OF D_{125C} VALUES FOR 103 SPORE ISOLATES FROM MARINER 69.

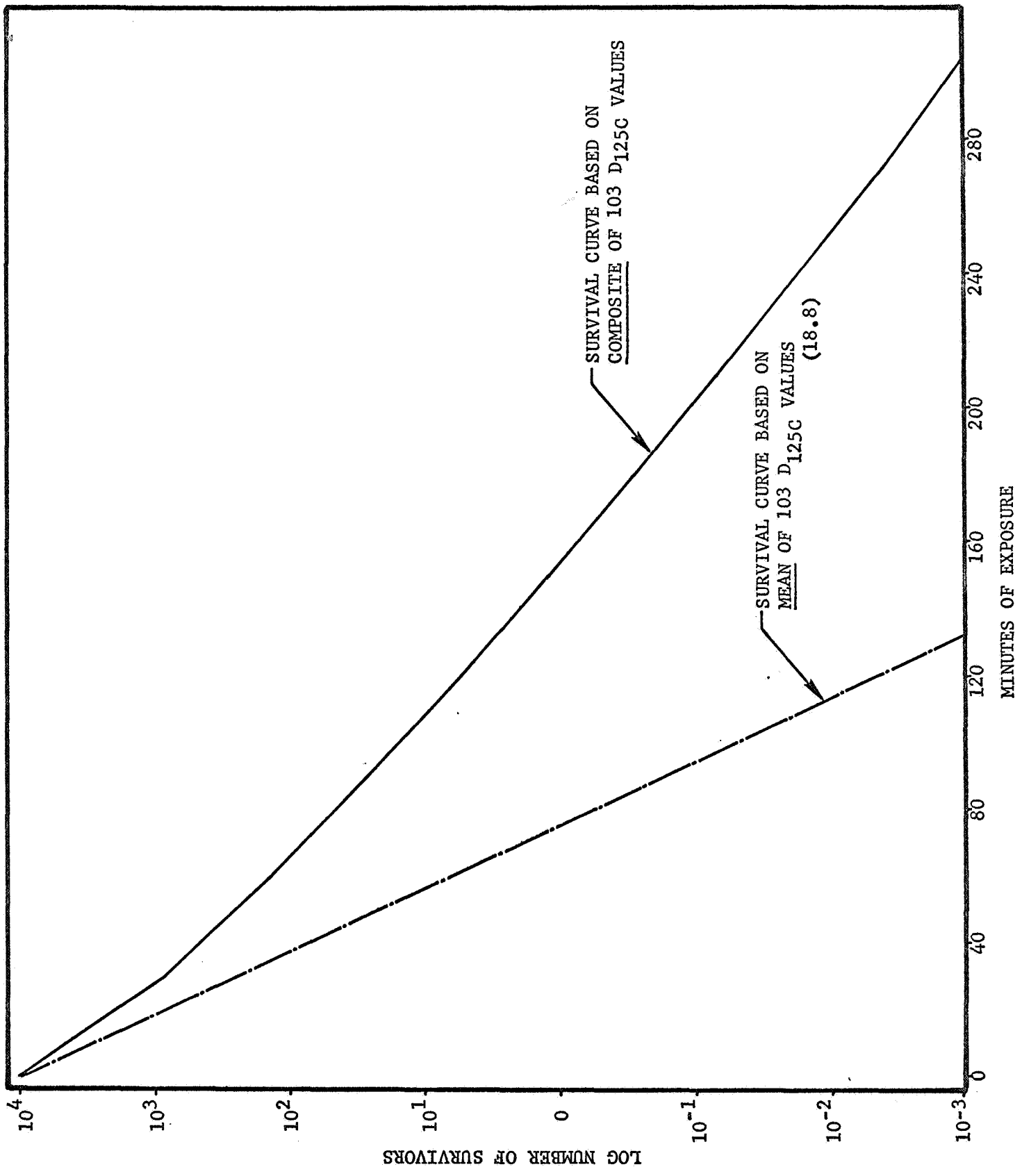

FIGURE 2. SURVIVAL CURVES BASED ON A FREQUENCY OF D_{125C} VALUES FOR 103 SPORE ISOLATES FROM MARINER 69.